

Validation in the Clinical Diagnostics Laboratory

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Desirable Total Error, Imprecision, and Bias

“Current databases on biological variation: pros, cons and progress.” , Ricos, Scand J Clin Lab Invest 1999; 59:491-500 from <http://www.westgard.com/biodatabase1.htm>

Analyte	Biological Variation		Desirable specification		
	CVw	CVg	I(%)	B(%)	TE(%)
α 1-Antitrypsin	5.9	16.3	3.0	4.3	9.2
Amyloid A	25.0	61.0	12.5	16.5	37.1
C reactive protein	42.2	76.3	21.1	21.8	56.6
C3 Complement	5.2	15.6	2.6	4.1	8.4
CA 125 antigen	24.7	54.6	12.4	15.0	35.4
Carcinoembryonic antigen (CEA)	12.7	55.6	6.4	14.3	24.7
Thyroglobulin	14.0	39.0	7.0	10.4	21.9

CVw = within-subject biological variation,

CVg = between-subject biological variation,

I = desirable specification for imprecision,

B = desirable specification for inaccuracy,

TE = desirable specification for allowable total error

Relevant Guidance

FDA bioanalytical method validation guidance, may 2001 (BMV)

White paper – 2012 white paper on recent issues in Bioanalysis and alignment of Multiple Guidelines – DeSilva et al., Bioanalysis, 2012, 4 (18), p2213 – 2226

CLIA/CAP Checklist for Clinical Chemistry and Toxicology (Updated 7/29/2013)

CLSI guidance documents (EP 5,6,7,9,10,14,15,17 and 28)

Acceptance Criteria: LC-MS/MS (4/6/15/50% - BMV)

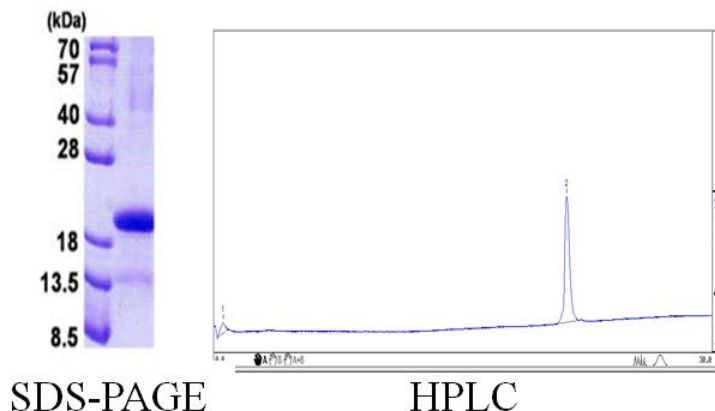
20% Inaccuracy and Imprecision @ LLOQ, 15% throughout the range
2/3 of all samples meet criterion, at least 50 % of each level meets criterion

Acceptance Criteria: Immunoassay (4/6/20/50% - BMV)

25% Inaccuracy and Imprecision @ LLOQ, 20% throughout the range
2/3 of all samples meet criterion, at least 50 % of each level meets criterion

Standardization without Gravimetry

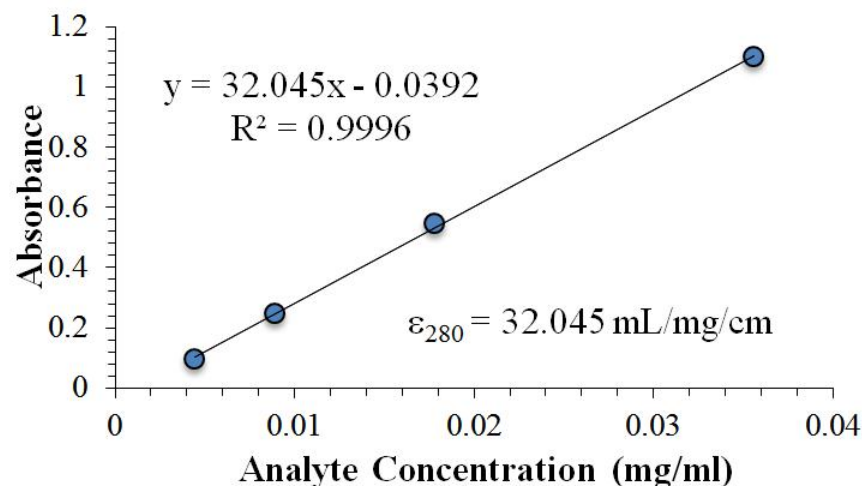
Protein/Peptide Purity



Amino Acid Analysis

Replicate	Measured Concentration (ug/mL)	
	Analyte	BSA QC
1	515.87	1833.55
2	504.36	1820.26
3	483.63	1853.48
Average	501.29	1835.76
%CV	3.26%	0.9%
% Bias	-	-8.2%

Spectrophotometry



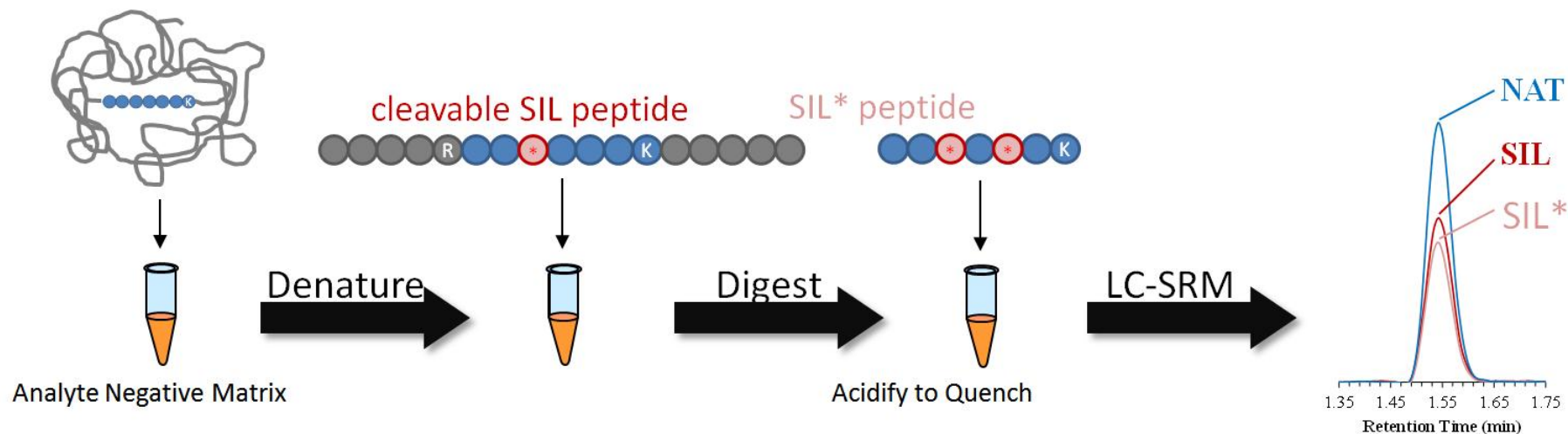
Master Stock:

1. Confirm purity by HPLC and/or SDS-PAGE
2. Assign concentration by AAA
3. Generate Extinction Coefficient at $\lambda = 280\text{nm}$

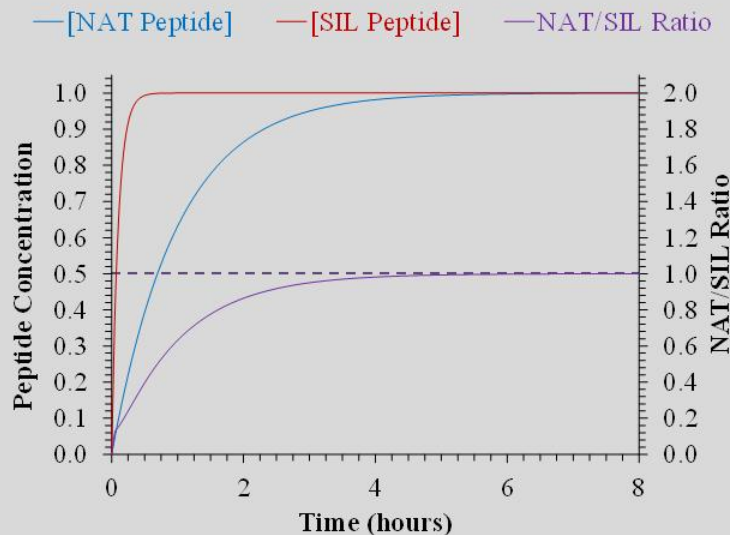
New Stocks:

1. Confirm purity by HPLC and/or SDS-PAGE
2. Assign concentration Absorbance at $\lambda = 280\text{nm}$

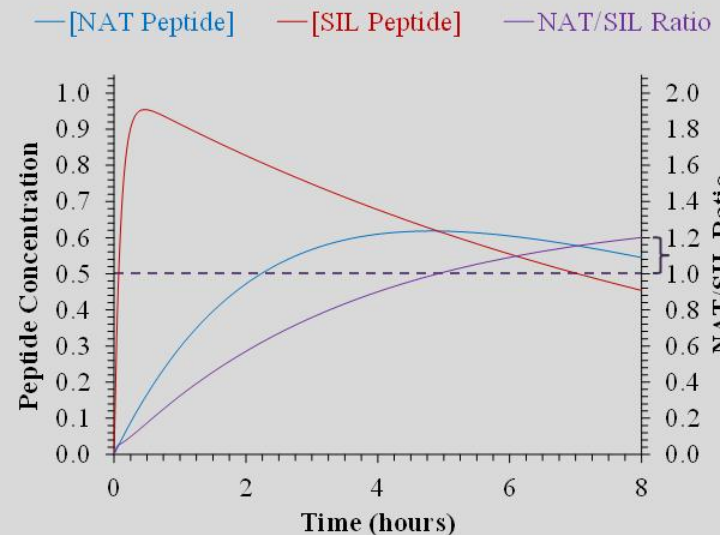
Calibration, Internal Standardization & Digestion Kinetics



Pseudo Matrix (Calibrator)

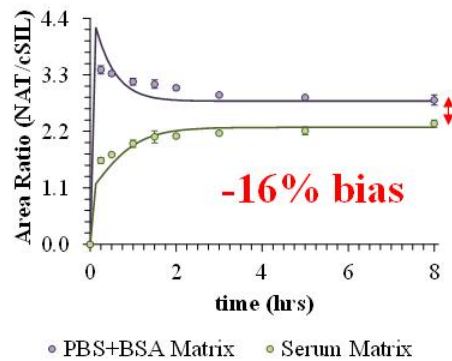


True Matrix (Sample)

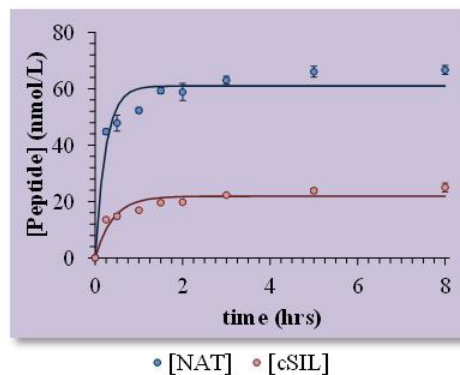


Calibration, Internal Standardization & Digestion Kinetics

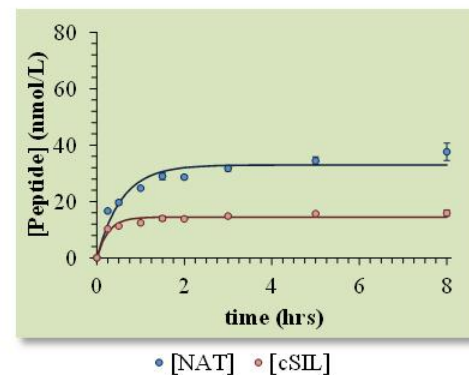
TG Peptide 1
2 M Urea



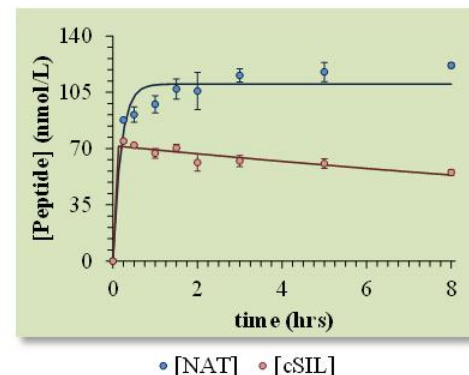
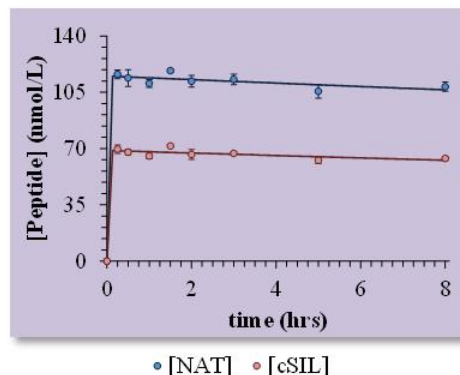
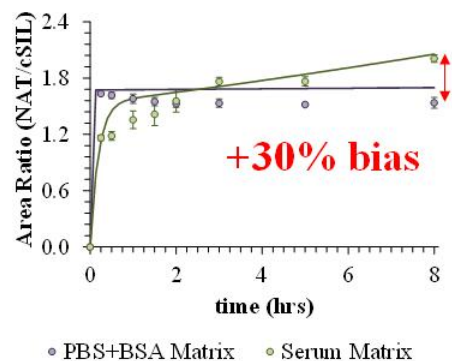
PBS + 0.5%BSA



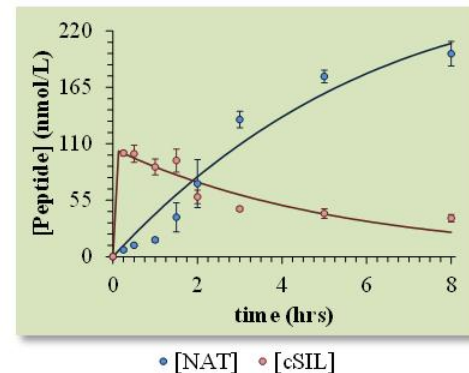
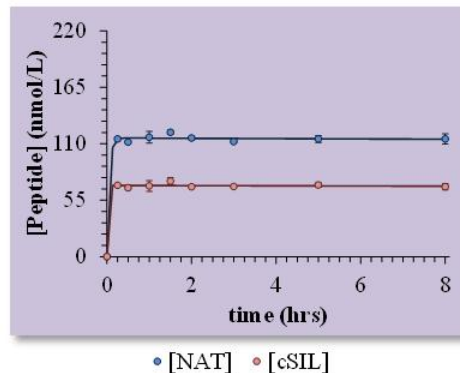
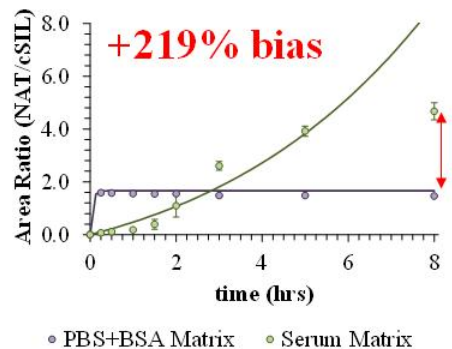
Negative Serum



TG Peptide 2
2 M Urea



TG Peptide 2
0.2% DOC

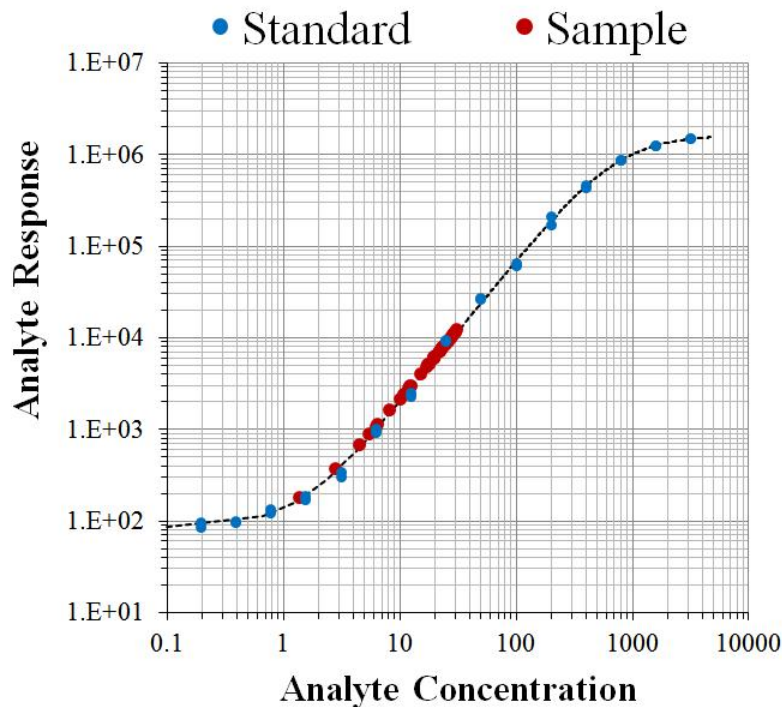


Calibrators (EP6)

Nature: Samples with known concentrations of target analyte(s)
Describe the analytical measurable range of the assay

Matrix: Matched to the sample type (Exogenous)
Proxy-similar to sample type (Endogenous)
Dissimilar- Solvent based (Endogenous where no proxy matrix is viable)

Development Phase

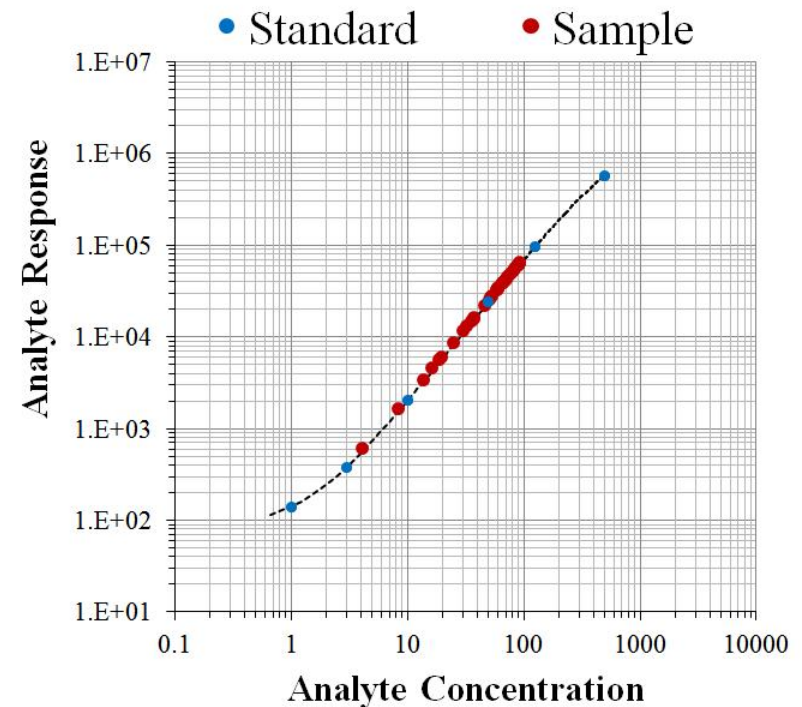


Refinement

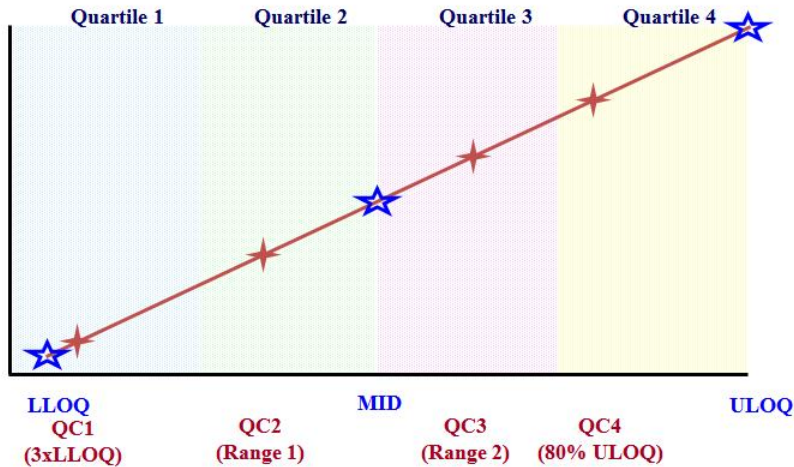


Dil. Factor
High Std
Low Std
(Anchoring)

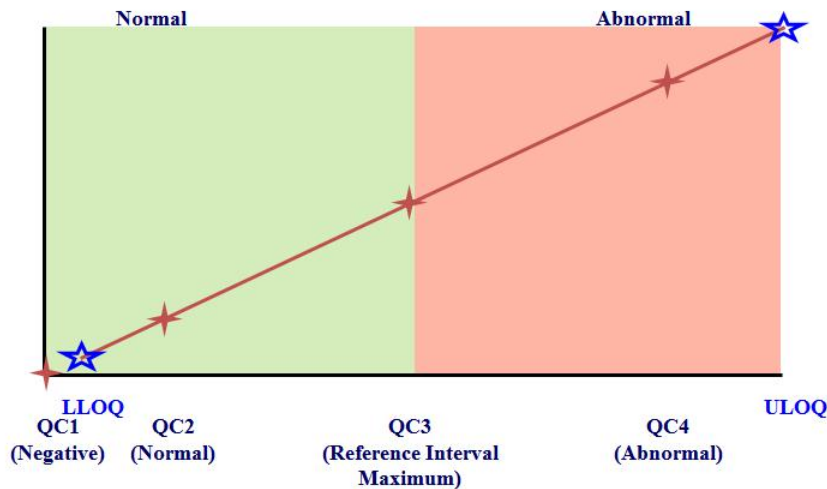
Validation Phase



Accuracy and Precision – FDA BMV and CAP



Proxy matrix samples at LLOQ, MID and ULOQ
– Accuracy and Precision
Samples at 3xLLOQ, 80% of ULOQ, quartiles 2&3 or reference ranges – **Endogenous Precision ONLY** – unless higher order accuracy target,
Exogenous - Accuracy and Precision



Calibration Curve = Analytical Measureable range (AMR)
Minimum of 2 quantified liquid QC's at different levels (CLIA '88) – Negative, normal, reference interval cutoff and abnormal.

Accuracy Validation

Inter-assay Accuracy									
Org	FDA-IVD	FDA-GLP	CAP	CLIA '88	SAMHSA	EP-10A3	EP-15	EP-6	EP-17A**
Levels	2	5	2	2	75%/125% Cutoff	3 spanning the medically relevant range, Level 2 exactly halfway between 1 and 3. Must be a reference material for accuracy	Certified Reference material within AMR	NA	1 - 5 at or near LLOQ
Replicates	20	3x6	1	Undefined	Undefined	Prescribed Order: 2, 3, 1, 2, 2, 1, 1, 3, 3, 2	3	NA	40
Days	20	3	20	Undefined	Undefined	5 (Lab), 20 (Manufacturer)	5	NA	5
Intra-assay Accuracy									
Levels	2	5	2	2	75%/125% Cutoff	Undefined	NA	5 - 11	1 - 5 at or near LLOQ
Replicates	20	3x6	Undefined	Undefined	Undefined	Undefined	NA	2 - 4	40
Data Reduction	Bias	Bias	Bias	Bias	Bias	Bias	Bias, mean, CV, additional calculations (See appendix of EP-15)	Numerous Calculations (See EP-6)	Total Error (Bias + 2xSD for 95% CI, Bias + 4xSD for 99.5% CI)
Acceptance Criteria	*	15/20 % Rule	*	*	*	*	*	*	*

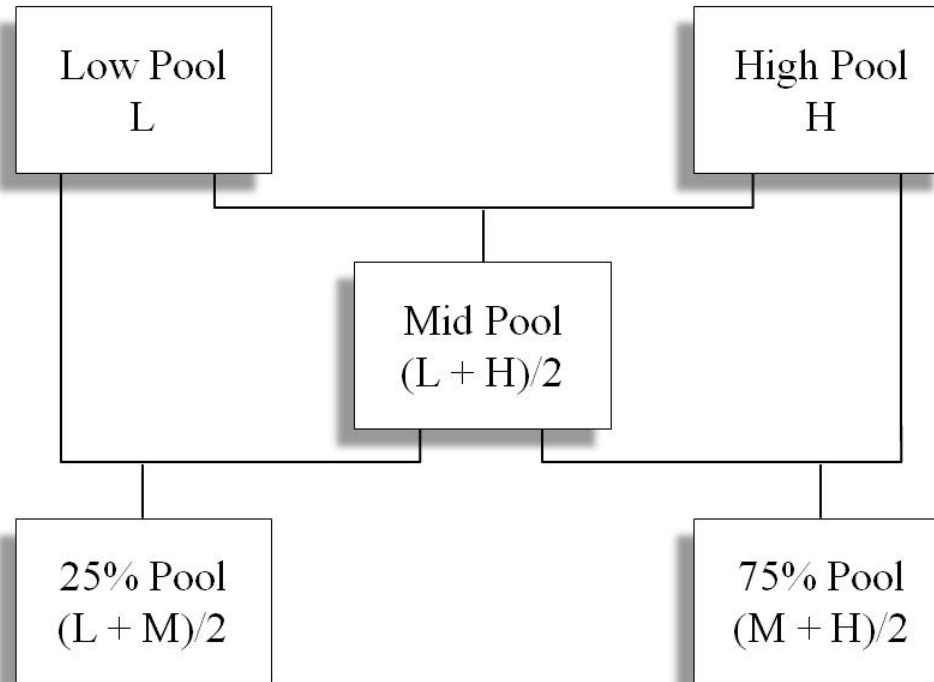
5 levels Exogenous, 2 levels Endogenous – 25 replicates in 1 day (intra), 1 replicate/level 25 runs (LC-MS/MS), 5 replicates/level over 5 runs (immunoassay)

Precision Validation

Inter-assay Precision								
Org	FDA-IVD	FDA-GLP	CAP	CLIA '88	SAMHSA	EP-10	EP-15	EP-5
Levels	2	3	2	2	75%/125% Cutoff	3 spanning the relevant range, Level 2 exactly halfway between 1 and 3	Minimum of 2 at Relevant Medical Decision Points	2
Replicates	20	3x6	1	Undefined	Undefined	Prescribed Order: 2, 3, 1, 2, 2, 1, 1, 3, 3, 2	3	2 batches per day separated by 2 hours
Days	20	3	20	Undefined	Undefined	5 (Lab), 20 (Manufacturer)	5	20
Intra-assay Precision								
Levels	2	3	2	2	75%/125% Cutoff	Undefined	Minimum of 2 at Relevant Medical Decision Points	2
Replicates	20	6	Undefined	Undefined	Undefined	Undefined	3 over 5 days	20
Data Reduction	CV	CV	CV	CV	CV	Multiple Regressions (See appendix of EP-10A3 for calculations)	Mean, CV, additional calculations (See appendix of EP-15)	Various Calculations (See EP5)
Acceptance Criteria	*	15/20% Rule	*	*	*	*	*	*

5 levels Exogenous and Endogenous – 25 replicates in 1 day (intra), 1 replicate/level 25 runs (LC-MS/MS), 5 replicates/level over 5 runs (immunoassay)

Relative and Absolute Recovery: EP7, EP10, FDA BMV



Relative Recovery/Accuracy:

Matrix Agnosticism: Calibrators/QC's/Samples – Equivalent digestion
Strategy also used for: Counter Ions/Collection tube Types,
Known clinical confounders (disease states/elevations of binding proteins)

Definitive Recovery/Accuracy:

External proficiency and/or inter-assay/laboratory correlations– CAP Checklist/EP10
Method of standard addition with Isotope dilution (3 levels)

Assay Validation Criteria and Acceptance part 1

Validation Process

Materials Requirements and Acceptance Criteria

Materials Selectivity

Blank matrix test 6+ Lots: Response<20% LLOQ in 5 of 6
Internal Standard: Response<20% LLOQ.

Sensitivity

Limit of blank or functional sensitivity - Back-fit Bias (25) and CV (25)

Standard Curve Fit

6-10 levels run in each assay, evaluated over 5 assays (days),
Back-fit Bias (20/15) and CV (20/15) except LLOQ

Carry-over

After ULOQ – 3 runs including <20% LLOQ
Stress test (10 -100x ULOQ), how many for <20% LLOQ

Dilutional Linearity

5 Level sample mixing – Bias/Precision (20/15)
1:1 – 1:50000 dilution - Bias/Precision (20/15)
Serial 100-10% dilution - Bias/Precision (20/15)

Recovery

ALL matrices and calibrators; min 3 spiking levels; Min 4
measurements per spike. Bias/Precision (20/15)

Accuracy (Intra and Inter-assay)

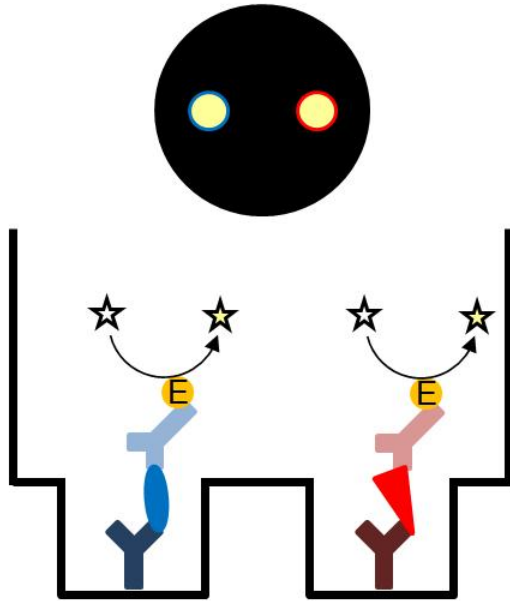
Min. 2 levels of QC's plus LLOQ and ULOQ; Min 25
measurements each in a single run and across 25 days. - Bias
20/15% and 25/20% @LLOQ

Precision (Intra and Inter-assay)

Min. 2 levels of QC's plus LLOQ and ULOQ; Min 25
measurements each in a single run and across 25 days. –
CV: 20/15% and 25/20% @LLOQ

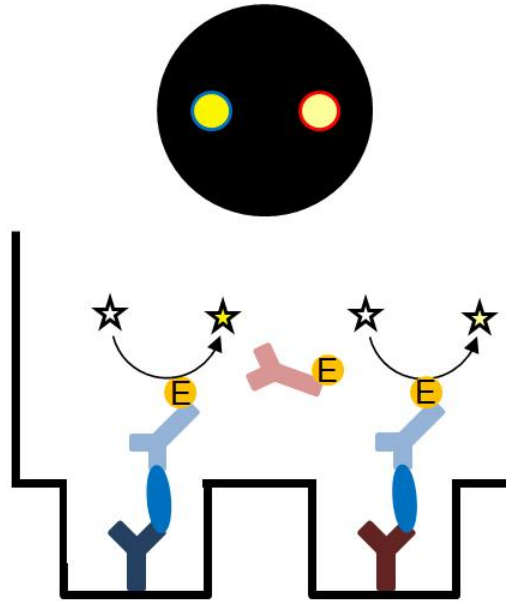
Specificity Testing in Multiplexed Immunoassays

Baseline Experiment



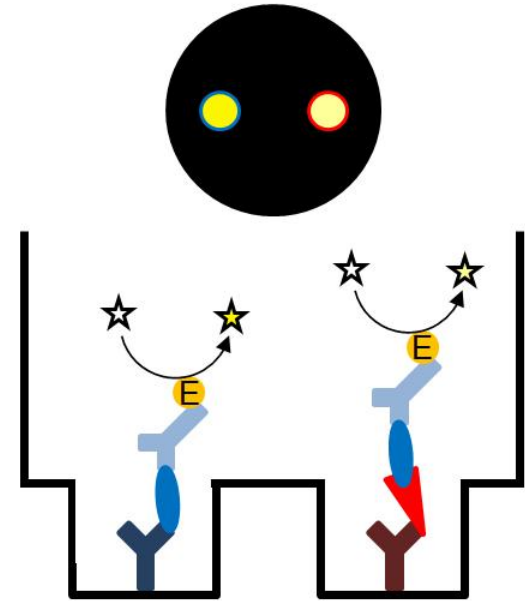
All Antibodies
All Analytes (LLOQ)

Experiment # 1



All Antibodies
One Analyte (ULOQ)

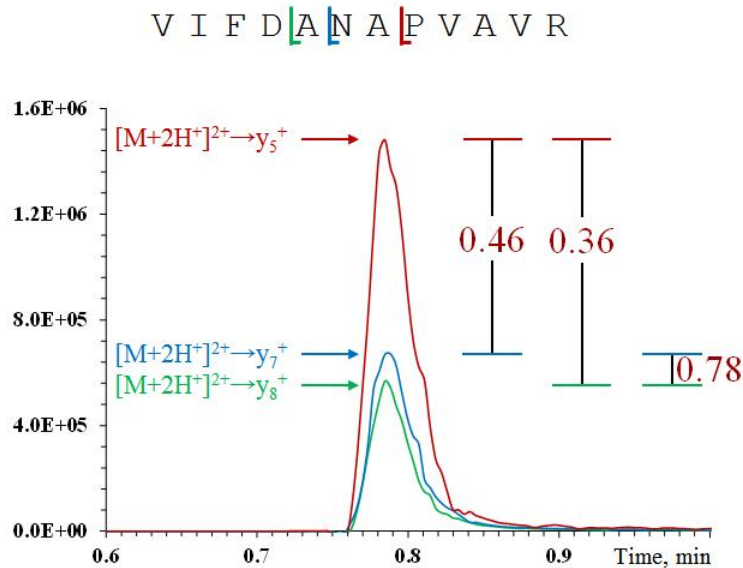
Experiment # 2



One Antibody
All Analytes (ULOQ)

Analyte	Measured Response		
	Baseline (LLOQ)	Experiment 1 (no Analyte)	Experiment 2 (no Antibody)
Protein A	63	59	32
Protein B	189	139	322

LC-MS/MS Transition Ratio Monitoring



Initially 3 peptides per protein and minimum of 3 transitions per peptide,

1 peptide may be sufficient with protein calibrator (*folded/biologically consistent state*)

BUT extensive selectivity and accuracy MUST be demonstrated

Transition Ratio Qualifier and Quantifier

Nature: Quality of reported result – Use of qualifying transition to add confidence

Design:

Measuring qualifying and quantifying transitions (both Analyte and IS) in distributed matrix/patient samples in a number of assay runs

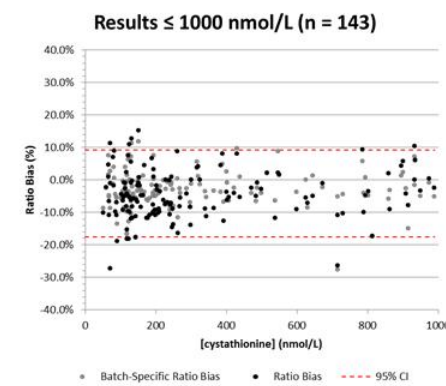
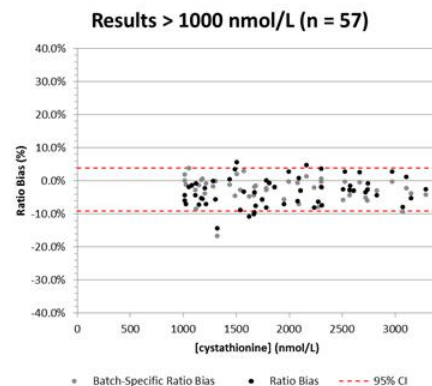
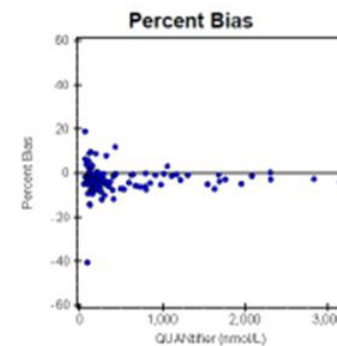
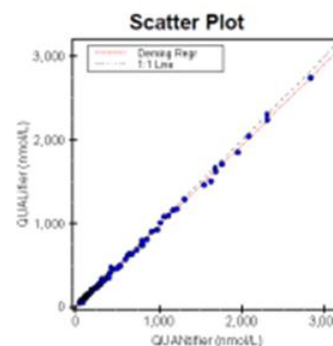
Quantify the assay using both transitions (**Bland-Altman – concentration variance vs concentration** **Top right**)

Determine the ratio of qualifier/quantifier peak areas (**Plot versus concentration, middle**)

Determine the appropriate ratio of qualifier/quantifier peak areas to use without pre-defined targets (**Bottom**)

Why?

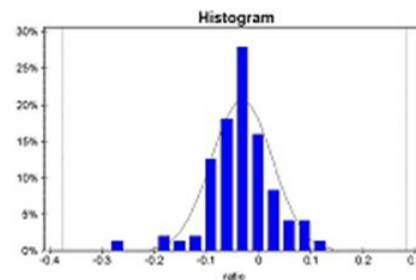
LC-MS/MS is “blind” to interferences, ratios enabled confidence. Intensity of qualifying ion often much lower near LLOQ



Reference Interval Estimation: Combined

	Central 95% Interval (N = 143)				Confidence Ratio
	Lower Value	90% CI	Upper Value	90% CI	
Nonparametric (CLSI C28-A)	-0.1753	-0.2763 to -0.1356	0.0922	0.0728 to 0.1192	0.35
Alternatives					
Parametric	-0.1569	-0.1705 to -0.1413	0.0869	0.0723 to 0.1015	0.12
Transformed Parametric	—	—	—	—	—

Confidence Limits for Nonparametric CLSI C-28A method computed from C28-A Table B.



Selection Criteria: None
 Bounds Filter: None
 Statistics:
 Mean: -0.03450
 SD: 0.06195
 Median: -0.03412
 Range: -0.2763 to 0.1192
 N: 143 of 143
 Distinct Values: 125
 Zeros: 0
 Central 95% Index: 3.6 to 140.4
 Analyst: QMB
 ExptDate: 20 Nov 2013

Matrix Constituents – CAP GEN.42030

Assurance™ Interference Test Kit from Sun Diagnostics New Gloucester, ME

Interferent Provided	Test Concentration	Typical Concentrate Values
Triglycerides	3000 mg/dl	>15,000 mg/dl (5X)
Hemolysate	500 mg/dl	>10,000 mg/dl (20X)
Total Protein (from Albumin and gamma-globulins)	12 g/dl	~25 g/dl (2X)
Bilirubin, conjugated	20 mg/dl	>400 mg/dl (20X)
Bilirubin, unconjugated	20 mg/dl	>400 mg/dl (20X)

Using Inter-assay Mean value as target and accounting for dilution, prepare (n=3)
Targets for LC-MS/MS shown, Immunoassay = 20% bias budget

Pool	Interferent Added	Target + Bias (%)
4 parts pool	1 part triglyceride	80 ± 15%
19 parts pool	1 part hemolysate	95 ± 15%
19 parts pool	1 part conjugated bilirubin	95 ± 15%
19 parts pool	1 part unconjugated bilirubin	95 ± 15%
1 part pool	1 part protein	50 ± 15%

Verification of Reference Intervals – CLSI C28-A3

Assay 20 + samples per Reference Interval, ensure 90 - 95% agreement

Verification of Reference Interval

Analyst: RPG

Date: 04 Sep 2012

Specimen Criteria: Children < 16 years

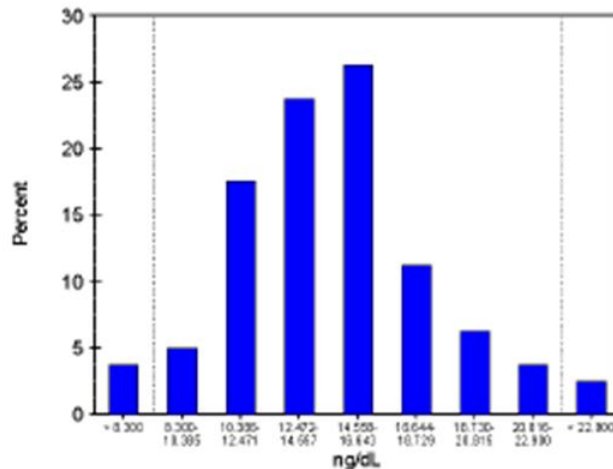
Reference Interval

Proposed	8.3 to 22.9 ng/dL
Results (total/excluded)	80 / 0
Max/Obs outside	10.0% / 6.3%
Passes	Yes

Statistical Analysis

Mean	14.9032 ng/dL
SD	4.4072
Median	14.5255
Range	2.836 to 37.882
Central 95% Interval	7.886 to 23.654
Central 95% Index	2.0 / 79.0

Reference Interval Histogram



Results Distribution

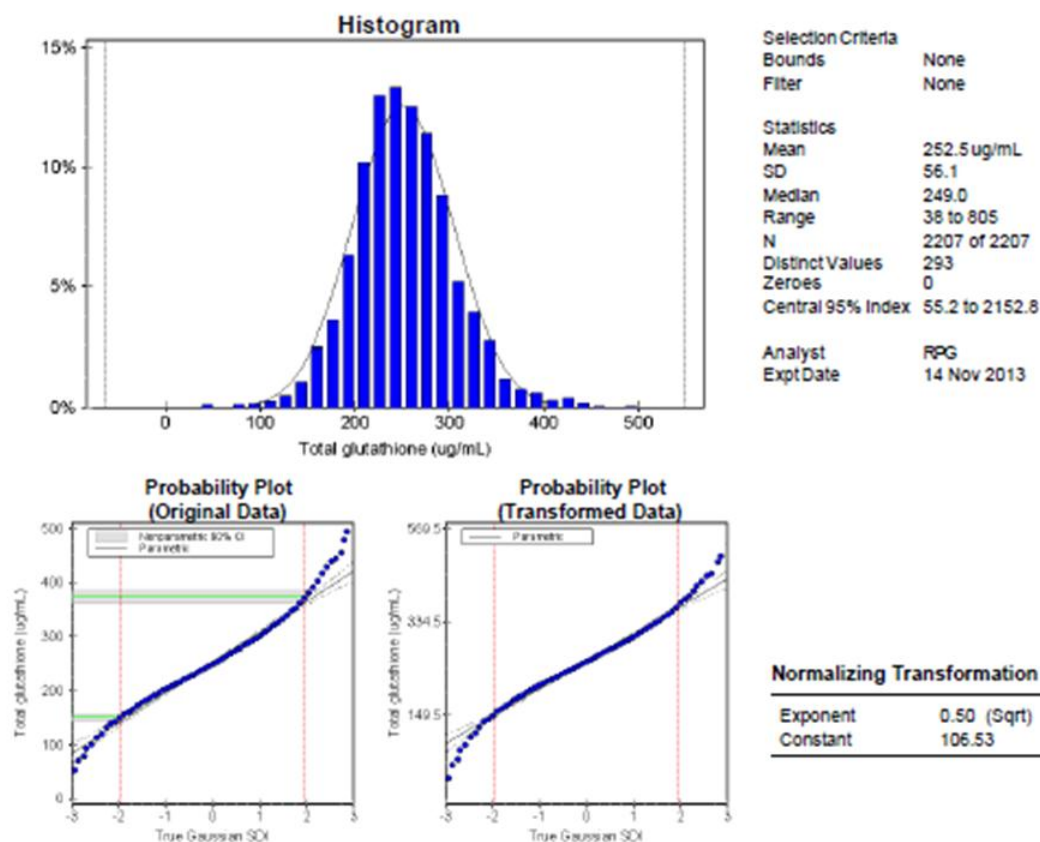
Interval	Percent	Count
< 8.300	4	3
8.300-10.385	5	4
10.386-12.471	18	14
12.472-14.557	24	19
14.558-16.643	26	21
16.644-18.729	11	9
18.730-20.815	6	5
20.816-22.900	4	3
> 22.900	3	2

Generation of Reference Intervals – CLSI C28-A3

Assay 120 + samples per age/gender, determine the middle 95% confidence interval to define “normal”

Central 95% Interval (N = 2207)					
	Lower		Upper		Confidence
	Value	90% CI	Value	90% CI	Ratio
Nonparametric (CLSI C28-A)	153	143 to 157	374	363 to 385	0.08
Alternatives					
Transformed Parametric	150	147 to 153	367	363 to 371	0.03
Parametric	143	139 to 146	362	359 to 366	0.03

Confidence Limits for Nonparametric CLSI C-28A method computed by exact formula.



Assay Validation Criteria and Acceptance part 2

Validation Process

Materials Requirements and Acceptance Criteria

Matrix Interference

Effect of lipid, Icteria and Hemolysis. Bias/CV % <15%/20%
Test anticoagulants Bias/CV % <15%/20%
Alternate sample types Bias/CV % <15%/20%
Deming regression slope 0.9-1.1, R >0.9 for same reference interval.

Batch Size

Maximum 184 samples LC-MS/MS. Bias % <15%/20%, Carry-over <20% LLOQ
Repeat samples n = 2 (first and 2nd plate) Deming regression 0.9-1.1, R >0.9

Transition ratio monitoring

Bias from extracted calibrators in each batch: Ideally <20% up to 3x LLOQ, < 15% after that for ratio > 0.5

Inter-assay Comparison

Min 40 – External Disparate assays – For information only
Internal assays same cals – Deming regression 0.9-1.1, R >0.9
Same assay different system – Deming regression 0.95-1.05, R >0.95
Same assay Manual/Automation – Deming regression 0.95-1.05, R >0.95

Reference Interval

120 in each reference interval
20 (95% CI) for range transfer 1/20 outside existing range

Sample Stability

All conditions tested from arm to final result
Mean Bias < 15%/20%@ LLOQ, %CV < 15%/20%@ LLOQ,
Deming regression 0.9-1.1, R >0.9

Materials Stability

All materials tested...stocks, cals, qc's, LC solvents etc...
Mean Bias < 15%/20%@ LLOQ, %CV < 15%/20%@ LLOQ,
Deming regression 0.9-1.1, R >0.9

Acknowledgements

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